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Barley- β -glucans reduce systemic inflammation, renal injury and aortic calcification through ADAM17 and neutral-sphingomyelinase2 inhibition

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In chronic kidney disease (CKD), hyperphosphatemia-induced inflammation aggravates vascular calcification (VC) by increasing vascular smooth muscle cell (VSMC) osteogenic differentiation, ADAM17-induced renal and vascular injury, and TNF α -induction of neutral-sphingomyelinase2 (nSMase2) to release pro-calcifying exosomes. This study examined anti-inflammatory β -glucans efficacy at attenuating systemic inflammation in health, and renal and vascular injury favoring VC in hyperphosphatemic CKD. In healthy adults, dietary barley β -glucans (B β glucans) reduced leukocyte superoxide production, inflammatory ADAM17, TNF α , nSMase2, and pro-aging/pro-inflammatory STING (Stimulator of interferon genes) gene expression without decreasing circulating inflammatory cytokines, except for γ -interferon. In hyperphosphatemic rat CKD, dietary B β glucans reduced renal and aortic ADAM17-driven inflammation attenuating CKD-progression (higher GFR and lower serum creatinine, proteinuria, kidney inflammatory infiltration and nSMase2), and TNF α -driven increases in aortic nSMase2 and calcium deposition without improving mineral homeostasis. In VSMC, B β glucans prevented LPS- or uremic serum-induced rapid increases in ADAM17, TNF α and nSMase2, and reduced the 13-fold higher calcium deposition induced by prolonged calcifying conditions by inhibiting osteogenic differentiation and increases in nSMase2 through Dectin1-independent actions involving B β glucans internalization. Thus, dietary B β glucans inhibit leukocyte superoxide production and leukocyte, renal and aortic ADAM17- and nSMase2 gene expression attenuating systemic inflammation in health, and renal injury and aortic calcification despite hyperphosphatemia in CKD.

In Chronic Kidney Disease (CKD), the development of hyperphosphatemia increases the risk of vascular calcification (VC) and cardiovascular mortality¹. Elevations in serum phosphate (P), even to levels below the upper normal limit, increase the propensity for VC indirectly by worsening secondary hyperparathyroidism (SHPT)

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and bone de-mineralization² and also directly, by inducing the osteogenic differentiation of vascular smooth muscle cells (VSMC)³.

Hyperphosphatemia also increases systemic inflammation^{4,5} and consequently, oxidative-stress-driven multi-organ injury^{6,7} worsening CKD-induced renal and vascular damage predisposing to VC and high mortality rates^{1,3}. Part of the renal and vascular deleterious effects of oxidative stress involves the induction of two critical enzymes: ADAM17 (A Disintegrin And Metalloproteinase, also called TACE for Tumor necrosis factor Alpha Converting Enzyme)^{8,9} and neutral sphingomyelinase 2 (nSMase2)¹⁰. Specifically, renal ADAM17 expression increases in CKD of all etiologies¹¹ aggravating renal damage¹² and systemic inflammation, the latter by releasing soluble TNF α to the circulation⁹. In turn, TNF α induces its own gene expression¹³, and also the ADAM17 gene¹⁴, generating a vicious ADAM17/TNF α feed-forward inflammatory loop that worsens multi-organ injury⁹. In fact, in the severe inflammation of LPS-induced endotoxic shock in mice, exclusive ablation of the ADAM17 gene in mouse myeloid cells is sufficient to markedly reduce mortality rates¹⁵.

In the vasculature, TNF α increases local inflammation aggravating VSMC osteogenic differentiation⁷, and also, nSMase2 gene expression and activity¹⁶, an essential determinant of aging-induced inflammation, atherosclerosis and VC^{10,16,17}. In fact, nSMase2 gene ablation or pharmacological inhibition of nSMase2 activity markedly reduces age-enhanced inflammation in health¹⁰, the higher propensity for atherosclerotic lesions in the ApoE $^{-/-}$ mouse¹⁷ and, more significantly for CKD, the release of exosomes initiating medial calcification¹⁶.

Based upon current therapeutic limitations to lower hyperphosphatemia in CKD¹⁸, and considering that orally administered yeast β -glucans efficaciously reduce multi-organ injury and mortality rates in LPS-challenged rats,¹⁹ this study was designed to examine whether an anti-inflammatory strategy with β -glucans could effectively attenuate systemic inflammation in health, including the mediator of early pro-senescent/pro-inflammatory interferon like responses, activated by the cGAS/STING pathway of cytosolic dsDNA recognition²⁰ and also, renal and vascular injury predisposing to VC in a rat model of hyperphosphatemic rat CKD.

To test this hypothesis, among the multiple natural sources for β -glucans, we chose barley β -glucans (B β glucans), based upon the safety of the FDA recommended daily intake of 3 to 5 g for their cholesterol^{21,22} lowering capacity. Specifically, we evaluated the efficacy of dietary B β glucans to reduce: (a) Systemic inflammation in healthy adults with normal renal function; (b) Renal and aortic inflammation and the propensity for CKD progression and aortic calcium deposition in a rat model of hyperphosphatemic CKD, and (c) The contribution of B β glucans regulation of ADAM17 and nSMase2 expression to their anti-inflammatory/anti-calcifying actions *in vivo* and *in vitro* in VSMC.

Results

Human study. Systemic anti-inflammatory actions by dietary B β Glucans. The daily intake of 3 g of B β Glucans, as barley bread by 10 individuals with normal renal function during 4 weeks, decreased circulating leukocyte mitochondrial superoxide production by 50% in one week (Supplementary Fig. 1).

In the 5 volunteers that completed the 3 g daily intake of B β Glucans for one month, the 50% inhibition of leukocyte superoxide production in the first week remained up to the end (week 4) (Fig. 1a). Furthermore, dietary B β Glucans also decreased leukocyte median mRNA levels of three recognized inflammatory markers: ADAM17 (80% at week 1 and 94% at week 4; $p < 0.05$) (Fig. 1b), TNF α (64% (not significant) at week 1 and 80% at week 4, $p < 0.05$) (Fig. 1c) and nSMase2 (58% at week 1 and 77% at week 4; $p < 0.05$) (Fig. 1d). These B β Glucans actions in circulating leukocytes after 1 or 4 weeks of a daily intake occurred despite no increases in serum levels either of β -glucans or in markers of bone and mineral homeostasis (calcium, phosphate, PTH, soluble klotho) or of systemic inflammation (C reactive protein and pro-inflammatory cytokines, except for a significant reduction of γ -interferon by week 4) (Table 1).

Dietary B β Glucans-driven reductions in serum γ -interferon levels were paralleled by significant decreases in leukocyte STING mRNA ($r = 0.54$; $p = 0.05$; Fig. 1f). In fact, leukocyte STING mRNA levels decreased by 90% ($p < 0.01$) by week 4 (Fig. 1e) of dietary B β Glucans intake. Furthermore, this reduction strongly correlated with that of leukocyte nSMase2 gene expression ($r = 0.80$; $p = 0.001$; Fig. 1g).

Animal study. Renal anti-inflammatory actions by dietary B β Glucans. The administration of the high P diet containing B β Glucans to uremic rats had no effect on daily food consumption (20 g/day) or body weights (CKD: 234 ± 13 g to 248 ± 23 g; $n = 13$; CKD + B β glucans: 237 ± 9 g to 255 ± 18 g; $n = 13$). Furthermore, despite undetectable serum (1,3)- β -D-glucan levels, the CKD + B β glucans group showed better renal function compared to the CKD group (lower serum creatinine, BUN, proteinuria and higher renal klotho) (Table 2), as well as reduced inflammatory markers (lower renal inflammatory cell infiltration (Table 2), ADAM17 protein and mRNA (Fig. 2a,b) and nSMase2 mRNA) (Fig. 2c). Serum and urinary TNF α levels were undetectable in both dietary groups.

Regarding bone and mineral parameters, there were no significant differences in serum calcium, phosphate, PTH, 25-hydroxyvitamin D, FGF23 and bone alkaline phosphatase between groups (Table 3). The higher renal klotho found in the CKD + B β glucans group (Table 2) did not result in increases in 24 hours phosphaturia (Table 3).

Animal study. Vascular anti-inflammatory actions by B β Glucans. The uremic rats fed the high P diet with B β glucans had significantly lower median aortic ADAM17 protein (47%) (Fig. 3a,b), ADAM17 and TNF α mRNA levels (80%) (Fig. 3c) and nSMase2 mRNA levels (65%) and activity (55%) compared with the CKD group (Fig. 3d). Furthermore, in the aortas of these uremic rats, TNF α mRNA levels correlated directly with ADAM17 mRNA ($r = 0.81$; $p < 0.001$) and also with nSMase2 mRNA levels and activity ($r = 0.89$; $p < 0.001$ and $r = 0.81$; $p < 0.01$, respectively).

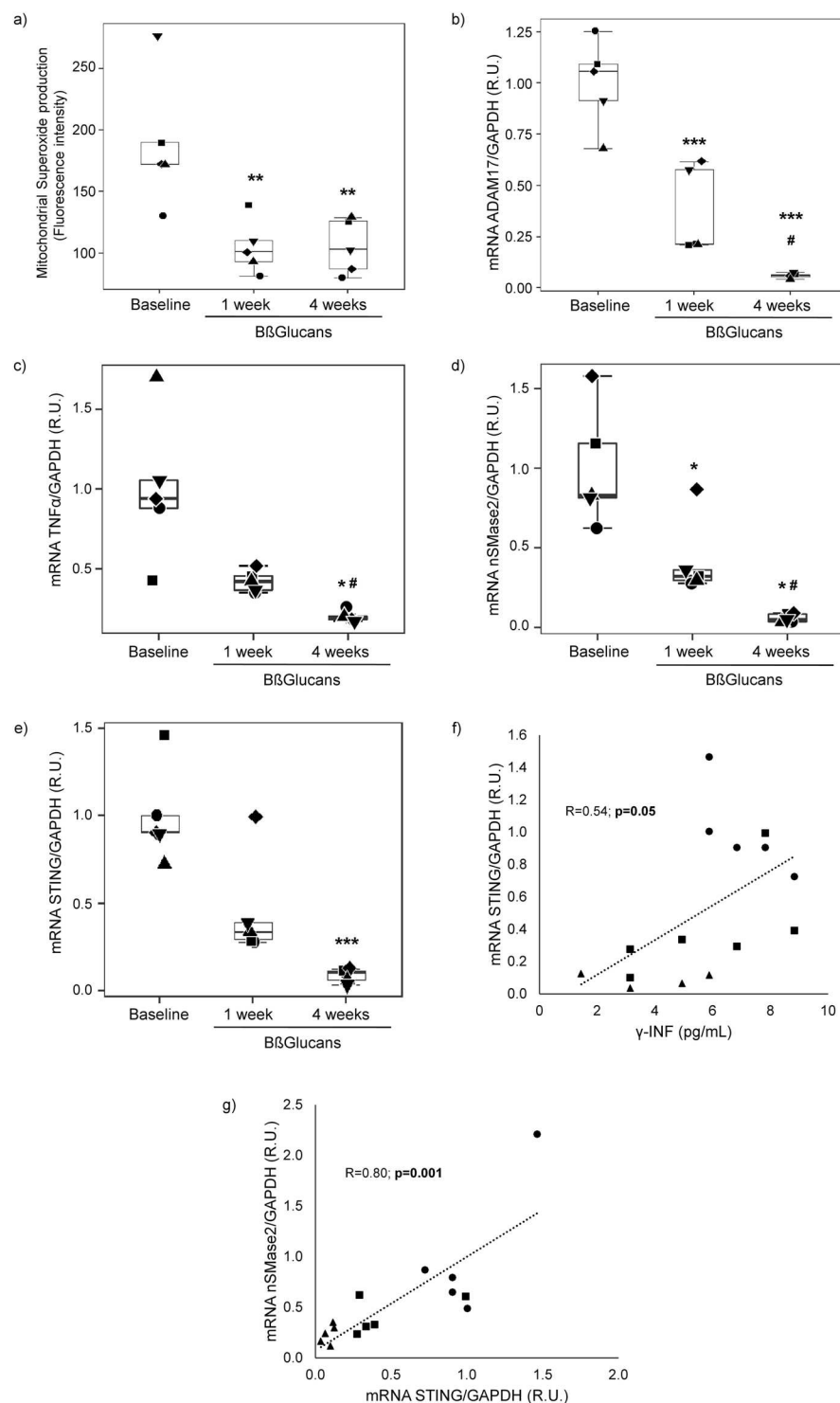


Figure 1. Dietary Bβglucans inhibit mitochondrial superoxide production, ADAM17, TNFα, nSMase2 and STING gene expression in circulating leukocytes from healthy adults. Box plot analyses of changes in baseline superoxide production (a), ADAM17 (b), TNFα (c), nSMase2 (d) and STING (e) mRNA levels (each shape represents one individual) in peripheral blood monocytes from 5 healthy adults ingesting 3 g of Bβglucans daily, as slices of barley bread, during 4 weeks. * $p < 0.05$ and ** $p < 0.01$ vs. Baseline, # $p < 0.05$ vs. 1 week. Correlation between leukocyte STING mRNA and serum γ-interferon levels (f) and between leukocyte STING and nSMase2 mRNA levels (g). Each shape represent baseline (●), one week (■) and 4 weeks (▲) values.

Animal study. Vascular anti-calcifying actions by dietary BβGlucans. In the CKD group, 36% of the aortas were Von Kossa positive while all aortas from the CKD + Bβglucans group stained negatively (Fig. 4a). Furthermore, median aortic total calcium was 8 times lower in the CKD + Bβglucans group ($7.5 \mu\text{g Ca/mg}$

	Baseline	1 week B β Glucans	4 weeks B β Glucans
	(n = 5)	(n = 5)	(n = 5)
Serum βglucans			
(1,3)- β -D-glucan (pg/mL)	Undetectable	Undetectable	Undetectable
Renal function			
Creatinine (mg/dL)	0.86 \pm 0.10	0.89 \pm 0.12	0.90 \pm 0.16
Urea (mg/dL)	23.9 [21.9–52.3]	26.3 [25.6–39.1]	26.8 [24.2–29.9]
Albumin (g/L)	46.02 \pm 2.57	45.3 \pm 1.48	44.46 \pm 2.15
Mineral homeostasis			
Calcium (mmol/L)	2.37 \pm 0.08	2.32 \pm 0.07	2.32 \pm 0.05
Phosphorus (mmol/L)	1.29 \pm 0.18	1.18 \pm 0.16	1.14 \pm 0.23
PTH (pg/dL)	56.00 [36.40–58.40]	51.70 [33.00–55.90]	31.9 [30.9–43.2]
Klotho (pg/mL)	925.33 [809.83–1,003.17]	873.17 [749.83–1,001.50]	868.00 [819.83–1,010.83]
Inflammation			
C Reactive Protein (mg/dL)	0.11 [0.04–0.22]	0.05 [0.04–0.10]	0.10 [0.04–0.13]
IL-2 (pg/mL)	1.37 \pm 0.21	1.54 \pm 0.26	1.47 \pm 0.48
IL-6 (pg/mL)	4.31 \pm 0.50	2.91 \pm 1.52	3.55 \pm 1.05
TNF α (pg/mL)	5.18 \pm 3.15	5.20 \pm 4.00	3.86 \pm 1.47
γ -INF γ (pg/mL)	7.05 \pm 1.28	6.31 \pm 2.28	3.71 \pm 1.73*
VCAM-1 (ng/mL)	2,180.68 \pm 117.97	2,130.27 \pm 34.16	2,246.75 \pm 139.80

Table 1. Serum biochemistries in healthy volunteers. Values indicate Mean \pm SD or Median (interquartile range). *p < 0.05 vs. Baseline.

	CKD	CKD + B β Glucans	p value
	(n = 13)	(n = 13)	
Serum Creatinine (mg/dL)	1.0 \pm 0.2	0.8 \pm 0.2	0.03
BUN (mg/dL)	52.9 \pm 9.6	44.3 \pm 7.2	0.02
Phosphaturia (nmol/24 hours)	1.9 \pm 0.5	2.3 \pm 0.6	0.15
Proteinuria (mg/24 hours)	101.1 \pm 80.0	21.4 \pm 32.1	0.04
α -Klotho (IOD/Area)	1,477.2 \pm 459.7	3,197.0 \pm 1839.4	0.02
Lymphocyte infiltration (% Area)	2.2 \pm 2.4	1.0 \pm 0.7	0.04

Table 2. Biomarkers of renal dysfunction in rat CKD. Values indicate Mean \pm SD.

protein) compared to uremic controls (CKD group: 60 μ g Ca/mg protein) (Fig. 4b). Despite the higher Ca deposition found in the CKD group, there were minor changes in median aortic mRNA levels of osteogenic differentiation markers (reductions in α -actin and increases in Runx2 and Osterix) compared to the CKD + B β glucans group, which did not reach statistical significance (Table 4). Instead, total aortic calcium content strongly correlated with increases in nSMase2 activity ($r = 0.60$; $p < 0.01$).

In vitro studies. Direct anti-inflammatory/anti-calcifying actions by B β Glucans in VSMC.

Protocol 1 searched for an effective anti-inflammatory dose of B β Glucans extracts in the murine monocyte cell line Raw 264.7, exposed to 5 μ g/mL of *E. Coli* LPS for 16 hours to reproduce severe systemic inflammatory stimuli.

Exposure to LPS increased mitochondrial superoxide production by 2-fold (Supplementary Fig. 1). A dose of 100 μ g/mL of commercial B β Glucans extracts was necessary to fully prevent LPS-induced superoxide production. In unstimulated monocytes, the 100 μ g/mL dose of B β Glucans extracts elicited anti-inflammatory actions as it reduced baseline monocyte superoxide production by 50% (Supplementary Fig. 1).

Protocol 2 examined the impact of LPS-driven inflammation on VSMC phenotype. In A7r5 cells, the exposure to 100 μ g of B β Glucans/mL for 16 hours had no effect on basal nSMase2, TNF α or ADAM17 gene expression (Fig. 5a–c). A7r5 exposure to LPS for 16 hours sufficed to significantly increase nSMase2, TNF α and ADAM17 gene expression (Fig. 5a–c) above the levels in unstimulated controls. Furthermore, the combination of the same dose of LPS with 100 μ g/mL B β glucans fully prevented LPS-driven increases in nSMase2 gene expression maintaining nSMase2 mRNA at the levels of the control group, failed to counteract LPS-induction of TNF α and decreased LPS-induced ADAM17 gene expression only marginally (Fig. 5a–c).

Regarding osteogenic differentiation, A7r5 exposure to 100 μ g of B β Glucans/mL for 16 hours had no effect on basal mRNA levels of α -actin, Runx2 or osterix. Instead, while LPS exposure significantly decreased A7r5 levels of α -actin and increased Runx2 and Osterix gene expression compared to those in the control group (Fig. 5d–f), the combination of LPS with 100 μ g/mL B β glucans attenuated the stimulatory effect of LPS maintaining the osteogenic differentiation markers at levels similar to those in the control group (Fig. 5d–f).

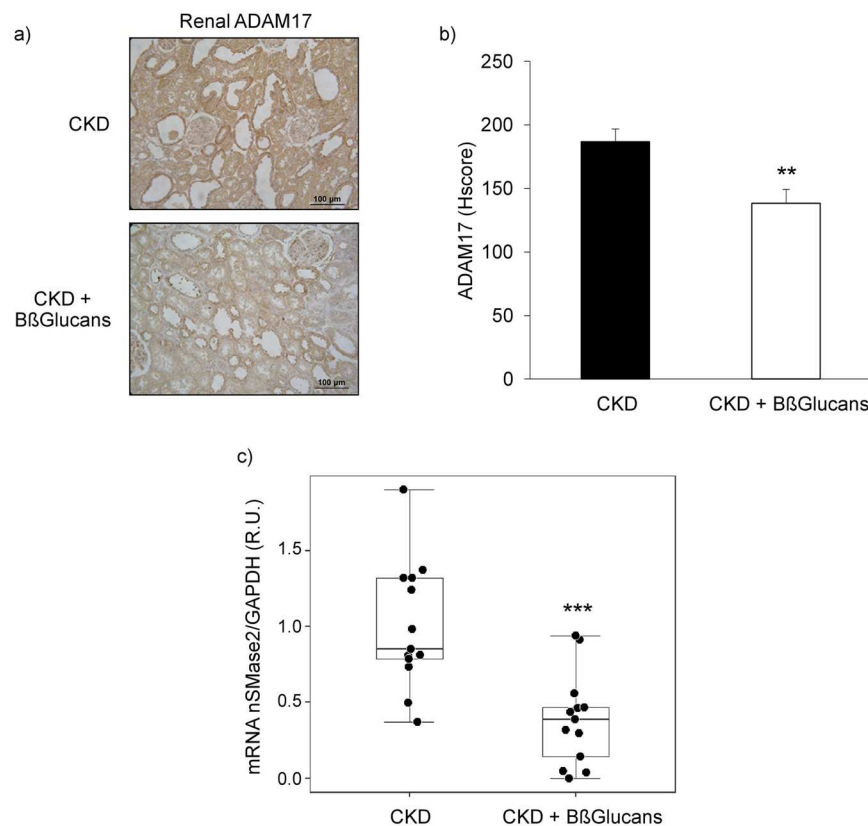


Figure 2. Renoprotection by dietary Bβglucans in hyperphosphatemic rat CKD. (a) Representative immunohistochemical images of renal ADAM17 from 5/6NX rats fed a high P diet with none (CKD; n = 13) or 2 mg of Bβglucans/g diet (CKD + Bβglucans; n = 13) during 4 weeks (Inset bars indicate relative scale); (b) Quantification of immunostaining. Bars and error bars represent mean ± SD of HistoScore values for all rats in both dietary groups; (c) Box plot analysis of nSMase2 gene expression in kidneys from rats described in (a). **p < 0.01 and ***p < 0.001 vs. CKD.

	CKD (n = 13)	CKD + Bβglucans (n = 13)	p value
Calcium (mg/dL)	9.8 ± 0.5	9.2 ± 0.8	0.19
Phosphate (mg/dL)	8.1 ± 2.6	7.7 ± 2.2	0.66
PTH (pg/mL)	3107 ± 2152	2044 ± 1127	0.14
25-hydroxyvitamin D (ng/mL)	29.4 ± 8.7	27.9 ± 6.5	0.63
FGF23 (ng/mL)	10.9 ± 5.6	12.5 ± 6.5	0.58
Bone alkaline phosphatase (U/mL)	42.1 (33.3–70.2)	28.1 (16.9–74.0)	0.28
(1,3)-β-D-glucan (pg/mL)	Undetectable	Undetectable	—

Table 3. Serum biomarkers of bone and mineral metabolism in rat CKD. Values indicate Mean ± SD or Median (interquartile range).

Protocol 3 examined the impact of uremia-driven inflammation on VSMC phenotype. The exposure of A7r5 to uremic serum from hyperphosphatemic rats for 16 hours significantly increased TNFα and ADAM17 gene expression. However, the almost 2-fold increase in nSMase2 gene expression was not statistically significant (Fig. 6a–c). By contrast, exposure of A7r5 cells to uremic serum + Bβglucans maintained the mRNA of the three markers of inflammation at levels similar to those in the cells exposed to control serum, which was not affected by exposure to Bβglucans (Fig. 6a–c).

In A7r5 cells exposed to normal serum, Bβglucans had no effect on basal levels of the osteogenic markers. Instead, the uremic serum only increased Runx2 gene expression with no significant changes in either α-actin or Osterix (Fig. 6d–f). However, the addition of 100 μg/mL of Bβglucans to the uremic serum prevented the increases in Runx2 gene expression maintaining its levels at basal values.

In vitro studies. Direct anti-calcifying and anti-nSMase2 actions by Bβglucans. In *Protocol 4*, A7r5 cells were exposed exclusively to calcifying media (CM: 2 mM Ca; 3 mM P) during 4 days, which increased

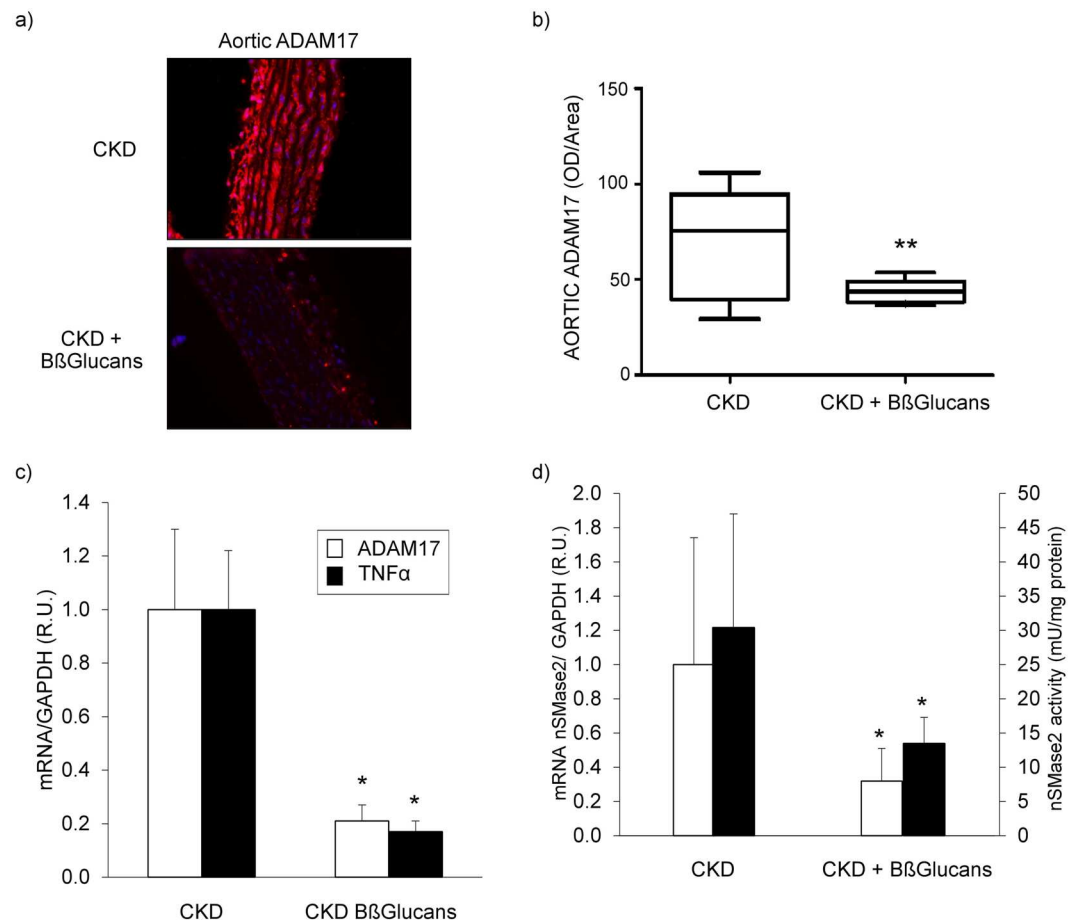


Figure 3. Anti-inflammatory vascular protection by dietary B β Glucans. **(a)** Representative immunostaining for ADAM17 (Inset bars indicate relative scale) in thoracic aortas from 5/6NX rats fed a high P diet with none (CKD; $n = 13$) or 2 mg of B β glucans/g diet (CKD + B β glucans; $n = 13$) during 4 weeks. **(b)** Quantification of aortic ADAM17 immunostaining. Box plots represent median (interquartile range) of OD/Area values for all rats in both dietary groups. **(c)** Aortic ADAM17 and TNF α gene expression; **(d)** Aortic nSMase2 mRNA (white bars; CKD $n = 9$; CKD + B β glucans $n = 7$) and activity (black bars; CKD $n = 13$; CKD + Barley $n = 11$). R.U.: Relative units. Bars and error bars represent mean \pm SD; * $p < 0.05$ vs. CKD.

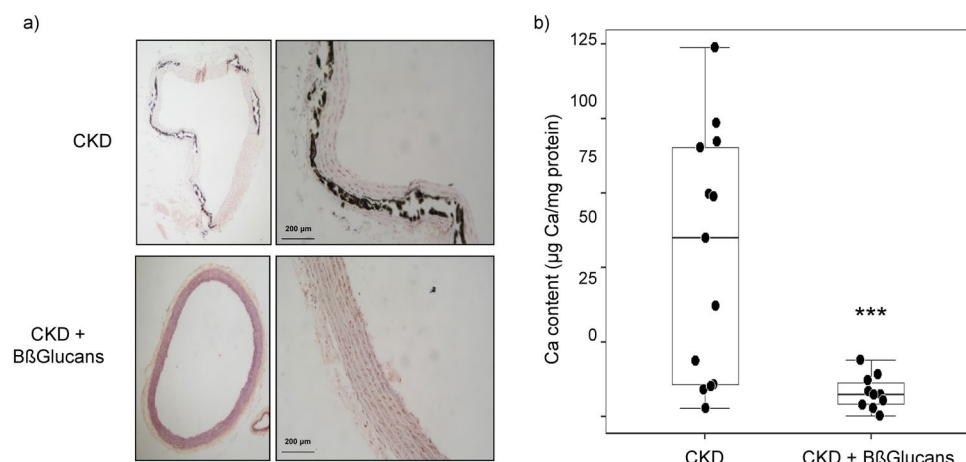


Figure 4. Anti-calcifying protection by dietary B β Glucans. **(a)** Representative calcium deposition measured by Von Kossa (black) staining (Inset bars indicate relative scale) in thoracic aortas from 5/6NX rats fed a high P diet with none (CKD; $n = 13$) or 2 mg of B β glucans/g diet (CKD + B β glucans; $n = 13$) during 4 weeks. **(b)** Quantification of calcium deposition in thoracic aortas described in **(a)**; boxplot analysis of changes in calcium content, * $p < 0.05$ vs. CKD.

	CKD	CKD + B β Glucans	p value
α -actin mRNA (R.U.)	0.97 (0.69–1.45)	1.20 (0.37–1.41)	0.696
	N = 12	N = 9	
Runx2 mRNA (R.U.)	1.67 (1.11–2.58)	1.51 (0.9–1.91)	0.481
	N = 10	N = 11	
Osterix mRNA (R.U.)	0.72 (0.47–1.13)	0.53 (0.47–0.73)	0.374
	N = 12	N = 9	

Table 4. Biomarkers of vascular osteogenic differentiation in rat CKD. Values indicate Median (interquartile range).

calcium deposition by 13-fold. The addition of B β Glucans to the CM markedly attenuated (by 72%) the increases in calcium deposition induced by the CM (Fig. 7a), prevented the decrease in α -actin and the increases in osteogenic Osterix and Runx2 mRNA levels (Fig. 7b).

Similar results were found in *ex vivo* experiments culturing aortic rings from normal rats under the same calcifying conditions used in A7r5 (data not shown).

In addition, in A7r5 cells exposed to CM, the activity of nSMase2 increased by 3-fold, an induction totally prevented by adding 100 μ g/mL of B β Glucans to the CM (Fig. 7c).

These direct anti-calcifying actions of B β Glucans extracts occurred in cells with undetectable mRNA levels of the β -glucan receptor Dectin 1 (not shown) and involved β -glucan internalization into A7r5 cells, as demonstrated by significant increases in intracellular B β Glucans levels from undetectable in cells exposed to the Non CM or CM alone to 2.3 ng/well ($p < 0.01$) in cells exposed to the CM + B β Glucans.

Discussion

This work presents unprecedented properties of dietary B β glucans of high translational relevance to attenuate systemic inflammation in health, as well as the progression of renal and vascular damage predisposing to VC in hyperphosphatemic rat CKD. These B β glucans actions extend beyond the inhibition of leukocyte superoxide production and of STING/interferon-like pro-aging/proinflammatory signals that precede any elevation of systemic inflammatory markers above normal levels, and involve a marked suppression of leukocyte, renal and vascular ADAM17 and nSMase2.

B β glucans control of systemic inflammation in health. In healthy adults, the daily intake of 3 g of B β glucans, recommended by the FDA to lower serum cholesterol^{21,22} could rapidly and efficaciously suppress systemic inflammation, a recognized inducer of VC. Indeed, dietary B β glucans markedly reduced circulating leukocyte superoxide production (50%) as well as leukocyte ADAM17 and nSMase2 gene expression (60%) after a week intake, despite unchanged serum levels of CRP, TNF α and several other inflammatory cytokines, all within the normal range, and undetectable serum β -glucans concentrations. Despite the small size of this population of adults with normal renal function, B β glucans rapid inhibition of these two pro-inflammatory genes in circulating leukocytes supports their potential to prevent/attenuate ADAM17-driven multi-organ injury and mortality rates⁹, and also nSMase2-driven age-enhanced inflammation regardless of serum TNF α ¹⁰ and the propensity for atheromatous lesions¹⁷, as conclusively demonstrated in experimental models of ADAM17 or nSMase2 gene ablation.

Significantly, by week 4, dietary B β glucans exerted a stronger (80 to 90%) inhibition of both ADAM17 and nSMase2 gene expression, and also markedly inhibited leukocyte TNF α mRNA levels, despite no further reductions of either of their inducers: superoxide production and serum TNF α . Thus, dietary B β glucans inhibition of leukocyte ADAM17, nSMase2 and TNF α gene expression involved mechanisms other than suppressing oxidative stress induction of ADAM17^{8,14} and nSMase2 gene expression^{10,23}, or TNF α -self up-regulation¹³. Significantly, among the inflammatory cytokines measured, dietary B β Glucans markedly reduced only circulating γ -interferon levels by week 4. This finding led us to evaluate whether dietary B β Glucans could also attenuate interferon-like pro-senescence/pro-inflammatory responses initiated by activation of the cGAS/STING pathway^{20,24,25}. We found that serum γ -interferon not only strongly correlated with leukocyte STING mRNA but, more significantly, dietary B β Glucans caused a 90% reduction of leukocyte STING mRNA by week 4. Thus, leukocyte STING emerged as a novel sensitive biomarker of leukocyte pro-aging/pro-inflammatory features, as its reductions by dietary B β Glucans precede any elevations above normal in circulating levels of inflammatory cytokines, including γ -interferon. Furthermore, the strong correlation between leukocyte STING and nSMase2 mRNA levels suggests that dietary B β Glucans' progressive and simultaneous suppression of leukocyte STING and nSMase2 gene expression could contribute to attenuate the age-enhanced inflammation attributed to increases in nSMase2 in health. Indeed, enhanced nSMase2 release of exosomes from leukocytes carrying a cGAS/STING-driven senescence/inflammation-associated secretory phenotype would propagate these signals to healthy neighboring cells²⁶. Even though the accuracy of these early biomarkers of the anti-aging, anti-inflammatory actions of B β Glucans was only identified in the 5 normal individuals who completed the 4 weeks of B β Glucans intake, the strength of the leukocyte STING/nSMase2 association ($r^2 = 0.65$) provides a solid base for early antinflammatory actions that need to be further corroborated in larger cohorts and long-term studies.

B β glucans renal and vascular protection in hyperphosphatemic CKD. In hyperphosphatemic rat CKD, a daily intake of 40 mg of B β glucans, as barley flour, effectively reduced inflammation-driven renal injury by targeting renal elevations in ADAM17, an enzyme induced by CKD of all etiologies¹² and also by TNF α ¹⁴ and oxidative stress⁸, despite undetectable serum β -glucan levels. The higher renal ADAM17 in uremic controls

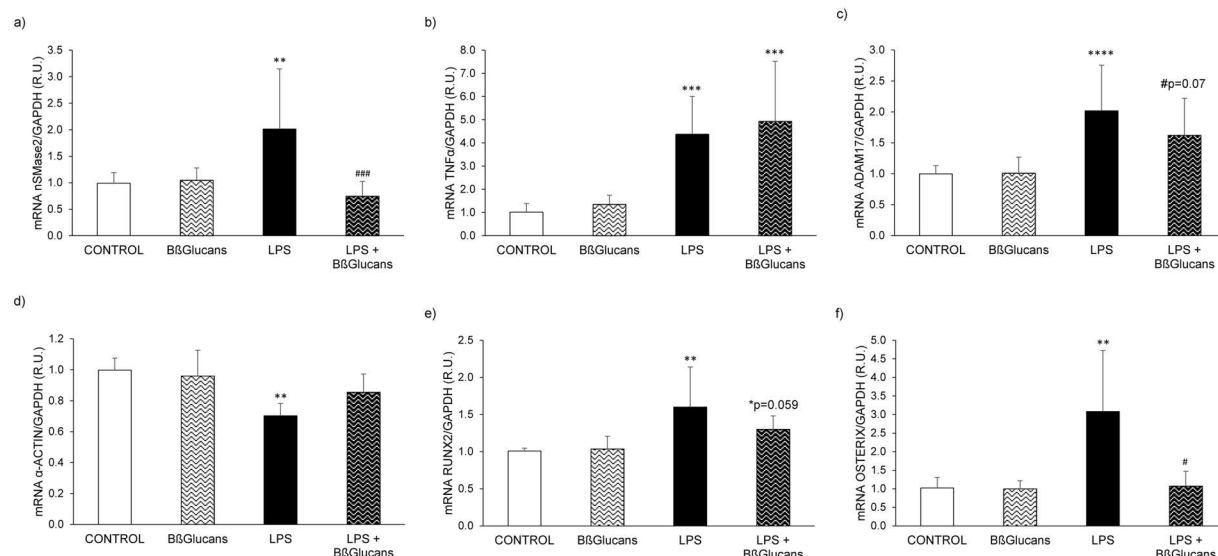


Figure 5. Bβglucans attenuate the vascular smooth muscle cell inflammation and osteogenic differentiation induced by LPS. Gene expression of nSMase2 (a), TNFα (b), ADAM17 (c), α-actin (d), Runx2 (e) and Osterix (f) in A7r5 cells exposed to control medium (CONTROL), 100 μg/mL of Bβglucans (β-Gl), 5 μg/mL of LPS (LPS) or the combination of 100 μg/mL of Bβglucans and 5 μg/mL of LPS (LPS + β-Gl) for 16 hours. Bars and error bars represent mean ± SD from three independent experiments, each performed in triplicate per experimental condition. R.U.: relative units. **p < 0.01 and ***p < 0.001 vs. Control, #p < 0.05 and ###p < 0.001 vs. LPS.

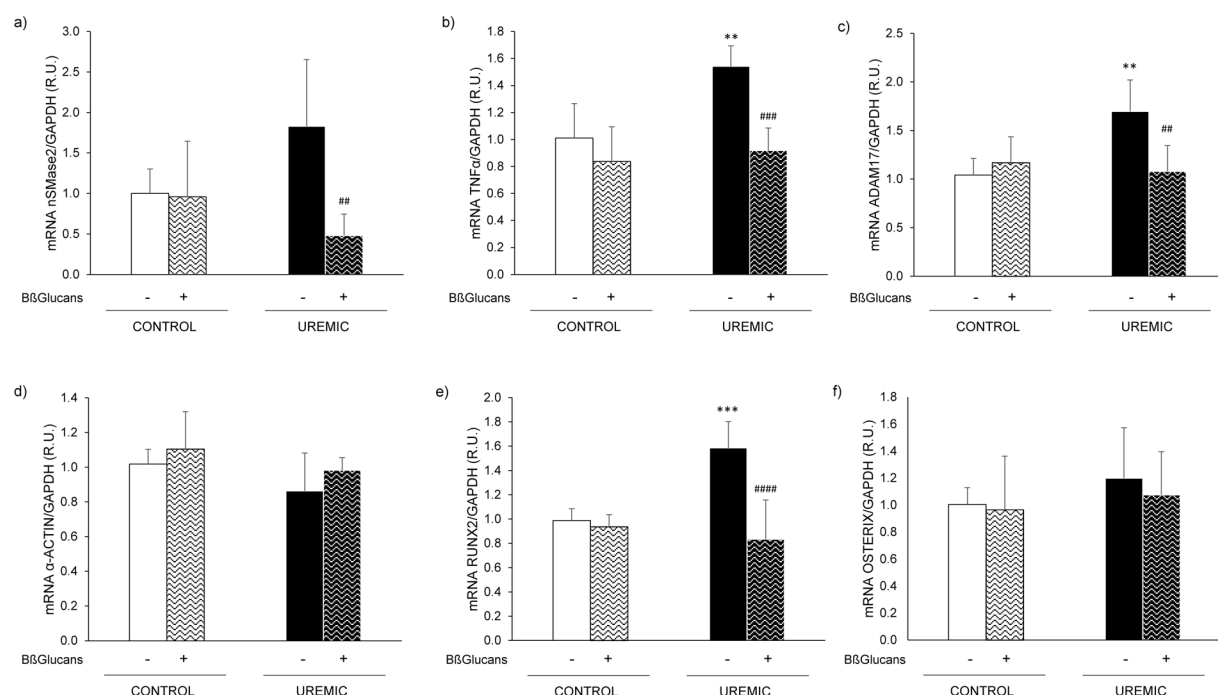


Figure 6. Bβglucans attenuate vascular smooth muscle cell inflammation and osteogenic differentiation induced by uremic conditions. Gene expression of nSMase2 (a), TNFα (b), ADAM17 (c), α-actin (d), Runx2 (e) and Osterix (f) in A7r5 cells exposed to serum from rats with normal renal function (Control), with or without 100 μg/mL of Bβglucans (β-Gl), or exposed to serum from rats with 14 weeks of uremia fed a high phosphorus diet (Uremic) with or without 100 μg/mL of Bβglucans for 16 hours. Bars and error bars represent mean ± SD from three independent experiments, each performed in triplicate per experimental condition. R.U.: relative units. **p < 0.01 and ***p < 0.001 vs. Control, #p < 0.01, ###p < 0.001 and ####p < 0.0001 vs. Uremic.

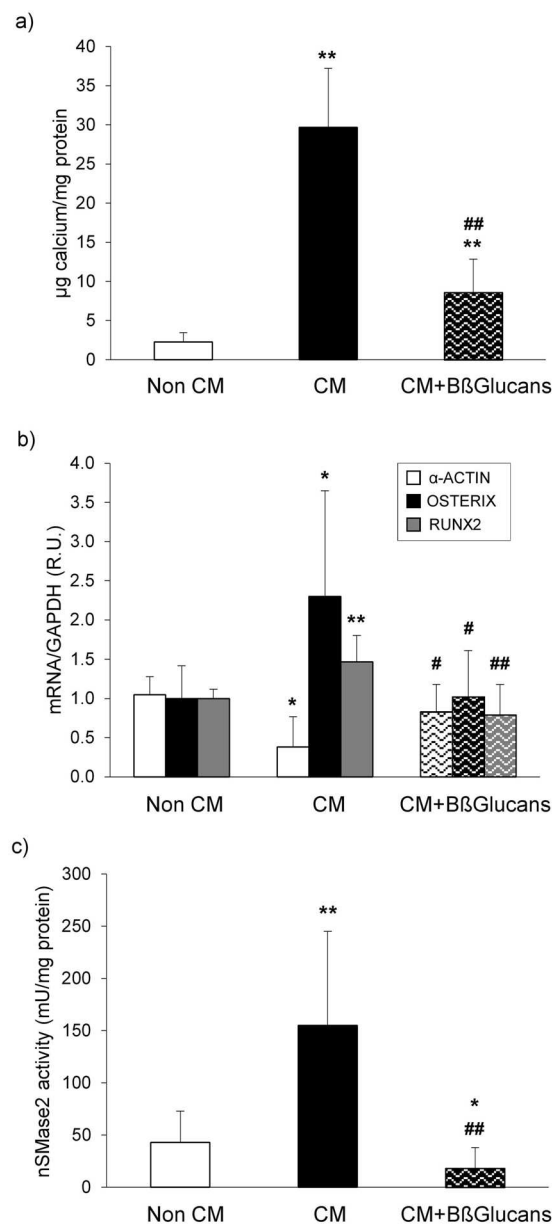


Figure 7. Bβglucans attenuate calcium deposition by inhibiting vascular smooth muscle cell osteogenic differentiation and nSMase2 activity. **(a)** Calcium deposition in A7r5 cells exposed to non-calcifying media (Non CM: 1 mM Ca; 1 mM P) or calcifying media (CM: 2 mM Ca; 3 mM P) with 0 or 100 μg/mL of Bβglucans (CM + β-Gl) during 4 days. **(b)** Gene expression of α-actin (white bars), osterix (black bars) and Runx2 (gray bars) in A7r5 cells treated as described. **(c)** nSMase2 activity in A7r5 cells treated as described. Bars and error bars represent mean ± SD from three independent experiments performed in triplicate per experimental condition. R.U.: relative units. * $p < 0.05$ and ** $p < 0.01$ vs. Non CM, # $p < 0.05$ and ## $p < 0.01$ vs. CM.

is sufficient to explain their increased inflammatory cell infiltration and 24 h proteinuria, as demonstrated by Lautrette and co-workers¹². In turn, the higher proteinuria of uremic controls could mediate the faster decline in GFR, as recently reported²⁷, and corroborated herein by the higher serum creatinine and BUN in the CKD group.

Dietary Bβglucans also reduced renal nSMase2 gene expression, which could extend the renoprotective benefits of this nutritional strategy to hypertensive individuals²⁸, by counteracting angiotensin II-driven-increases in renal nSMase2 or to diabetic nephropathy, by reducing the release to the urine of megalin loaded exosomes²⁹.

Bβglucans simultaneous attenuation of CKD progression and renal inflammatory cell infiltration, the two most potent down-regulators of renal α-klotho content^{5,30}, resulted in a higher renal α-klotho in the CKD + Bβglucans group. However, phosphaturia was similar between dietary groups, which suggested that the higher renal klotho was insufficient to ameliorate kidney resistance to FGF23 and, consequently, the deleterious renal impact of phosphate retention.

In the inflamed aortas from these uremic rats, TNF α mRNA levels correlated directly with ADAM17 mRNA, partly reflecting the expected TNF α induction of ADAM17⁹ and of its own gene^{13,14}, and also with nSMase2 mRNA levels and activity. Dietary B β glucans suppressed by 80% the self-perpetuating aortic ADAM17/TNF α -inflammatory loop⁹ and prevented the 3-fold elevations in nSMase2 reported to prompt pro-calcifying exosome release¹⁶, thereby reducing aortic calcium deposition in the CKD + B β glucans group by 8-fold compared to that in uremic controls. Interestingly, the higher calcium in uremic controls was unrelated to changes in the expression of markers of osteogenic differentiation and strongly correlated only with increased nSMase2. Thus, our aortic findings support the evidence that exosomes released by nSMase2 are the earliest calcified particles preceding overt calcification³¹, and also, that there are differential increases in vascular nSMase2 release of calcified and noncalcified exosomes causing heterogeneous calcium deposition at sites of early calcification^{31,32}. Furthermore, Von Kossa staining was positive only in 36% of the aortas from uremic controls supporting that overt calcification is a late event in the course of CKD. Significantly, dietary B β glucans fully prevented overt calcification, as none of the aortas in the barley group was Von Kossa positive despite no amelioration of systemic calcium and phosphate homeostasis.

Importantly, a B β glucans intake effective at reducing aortic nSMase2 gene expression and activity had no adverse impact on bone mineralization, as estimated by serum bone specific alkaline phosphatase levels. This is an important translational consideration to design therapeutic anti-nSMase2 strategies in CKD because the nSMase2 null mouse presents severe bone and dental mineralization defects^{33,34}.

Mechanisms for B β glucans' actions. An intriguing aspect of B β glucans potent anti-inflammatory, anti-ADAM17, anti-nSMase2, renal and vascular protective actions *in vivo*, is that they occurred with undetectable serum β glucans levels. Indeed, part of these actions may not require intestinal B β glucans absorption because oral B β glucans induce a saccharolytic shift in the microbiota that augments serum levels of anti-inflammatory short chain fatty acids, and also reduces circulating levels of p-cresyl-sulfate³⁵, a uremic toxin promoting renal and cardiovascular injury^{36,37}.

However, our studies *in vitro* in murine monocytes and VSMC exposed to commercially available B β glucans extracts (95% purity) demonstrated direct anti-inflammatory, anti-calcifying actions that support the pharmacokinetics of dietary B β glucans absorbed in the gut^{38–40}. Specifically, in murine monocytes, we first identified that a dose of 100 μ g/mL of B β glucans extracts was necessary to prevent the 2-fold increases in mitochondrial superoxide production induced by a 16 h exposure to an LPS challenge (5 μ g/mL). Significantly, this anti-inflammatory dose of B β glucans also reduced by 50% the superoxide production in unstimulated monocytes, thus reproducing the 50% inhibition exerted in normal subjects after a week, or a month, of a daily intake of 3 g of B β glucans.

Furthermore, the exposure of A7r5 cells to LPS (5 μ g/mL for 16 h) corroborated that severe inflammation rapidly induced a switch to an osteogenic phenotype, as the increases in the mRNA levels for ADAM17, nSMase2 and TNF α were associated to decreases in α -actin and increases in the osteogenic markers Runx2 and osterix. Importantly, the simultaneous exposure of A7r5 cells to LPS and the effective anti-inflammatory dose of B β glucans in immune cells, fully prevented LPS-induced inflammatory and osteogenic phenotypic changes without affecting VSMC baseline phenotype.

A7r5 cells also responded rapidly to uremic stimuli (serum from hyperphosphatemic rat CKD for 16 h) with significant elevations in ADAM17, TNF α and Runx2, but without significant increases in nSMase2 and osterix or decreases in α -actin. Nonetheless, B β glucans fully prevented all uremia-induced changes, thus maintaining baseline levels of all of these inflammatory/osteogenic markers. These findings support that a threshold for inflammatory or uremic stimuli is necessary to increase nSMase2 and osteogenic markers to levels resulting in overt calcification.

In VSMC exposed exclusively to prolonged (4 days) high Ca/high P conditions, B β glucans extracts decreased by 60% nSMase2 gene expression in the absence of any inflammatory or uremic stimuli, through a process involving β 1,3-glucan internalization into VSMC that is Dectin1-independent, as A7r5 cells showed undetectable Dectin1 mRNA levels. Several β -glucans receptors expressed in VSMC (Complement Receptor 3 or TLR2/6)^{41,42} could mediate B β glucans actions in A7r5 cells.

The internalization of (1,3) β -D-glucans into A7r5 cells exposed to B β glucans extracts may also occur in other B β glucans target cells, which could explain in part the systemic, renal and vascular anti-inflammatory/anti-nSMase2 actions of dietary B β glucans in healthy adults and in hyperphosphatemic rat CKD despite undetectable serum levels. Indeed, there is evidence that soluble β 1,3-glucose polymers, generated from oat and barley grains by the highly predominant β 1,3- β 1,4-glucanase in human gut bacteria⁴³, are internalized and further processed by intestinal epithelial and immune cells to shorter β 1,3-glucans. In turn, the shorter β 1,3-glucans released to the circulation³⁹ can be re-internalized into immune cells reducing serum levels while enhancing leukocyte systemic anti-oxidant/anti-inflammatory capacity^{41,44}, as demonstrated herein in healthy adults.

Barley is the least expensive cereal grain. Therefore, dietary B β glucans health benefits could reach the poorest populations of CKD patients and of healthy adults with systemic pro-aging or inflammatory problems and, therefore, at a higher risk for renal and vascular injury.

Prospective clinical trials are mandatory to evaluate the sensitivity and accuracy of leukocyte ADAM17, nSMase2 or TNF α mRNA and STING levels to personalize dietary B β glucans interventions for efficacious systemic, renal and vascular protection in the general population and to attenuate CKD progression and the initiation of medial calcium deposition in human CKD.

Material and Methods

Ethics statements. Approval for the human study was obtained from the Institutional Review Board (Comité de Ética en Investigación Clínica del Hospital Universitario Central de Asturias), in compliance with the Declaration of Helsinki. All participants gave a written informed consent prior to their inclusion in the study.

Approval for the animal study was obtained from the Ethics Committee for Animal Experimentation at Lleida University in compliance with current international legislation for animal research.

All methods in the experimental protocols described below were carried out following the regulations for biomedical research of the University of Lleida and the University of Oviedo.

Human study. Ten volunteers (2 men, 8 women, age 20 to 60 y.o.), with normal renal function ingested daily during 4 weeks barley bread, manufactured with flour from barley grains selected for their high (1–3) (1–4)- β -D-glucans content (8%), to provide 3 g of B β Glucans (produced at Food Science and Technology Department, Lleida University). Blood (5 mL) was drawn at baseline and at week 1 and 4 to obtain plasma and circulating leukocytes (after red cell lysis). As systemic inflammation biomarkers, we measured leukocyte mitochondrial superoxide production by flow cytometry in freshly isolated leukocytes. In the five volunteers (1 man, 4 women) that complied with the daily intake for one-month, we also quantified the mRNA levels of TNF α , ADAM17, nSMase2 and STING, as described in the *in vitro* studies. Serum levels of (1, 3) β -D-glucans, cholesterol, glycemia, markers of renal function, calcium and phosphate homeostasis, and systemic inflammation were measured as specified in blood chemistries. The reason to abandon the study was the omission of the daily intake more than twice per week.

Animal study. Five-sixth nephrectomized (NX) female Sprague-Dawley rats (200–225 g) were fed during 4 weeks a high phosphate diet (HPD: 0.9% P; 0.6% calcium, Ca; Altromin) containing either 0 (CKD: n = 13) or 2 mg of B β Glucans/g diet (CKD + B β Glucans) from the barley flour described above, with no changes in dietary protein, P, carbohydrate or lipid content. The modified Megazyme method quantified final β -D-glucan content in the diet⁴⁵.

In vitro studies.

- I. B β Glucans anti-inflammatory actions were examined in the murine monocyte cell line Raw 264.7 and in the rat aortic VSMC line A7r5.
Protocol 1: Rested Raw264.7, synchronized at G0 by exposure to FBS-free DMEM medium for 6 h, were treated for 16 hours with 5 μ g/mL LPS from *Escherichia coli* 0111:B4 (L4391, Sigma-Aldrich), 100 μ g/mL of barley (1–3)(1–4)- β -D-glucans (G6513, Sigma-Aldrich; 95% purity) or both.
Protocol 2: A7r5 cells were exposed to 5 μ g/mL LPS, 100 μ g/mL of barley (1–3)(1–4)- β -D-glucans or both in DMEM + 1% foetal bovine serum (FBS) for 16 hours.
Protocol 3: To test uremia-driven inflammatory stimuli, A7r5 cells were exposed to either control serum (a pool from rats with normal renal function fed a normal P diet; DMEM + 10% control serum), or to uremic serum (a pool from rats with uremia of 14 weeks fed a high phosphorus diet; DMEM + 10% uremic serum), with or without 100 μ g/mL of B β glucans for 16 hours.
- II. B β Glucans anti-calcifying actions were examined in A7r5 cells and in aortic rings (1–2 mm) from normal rats.

Protocol 4: Aortic rings were washed in cold PBS containing P/S and then placed in fibronectin pre-coated (100 μ g/mL) 6-well plates (8 rings/well) with growing media. A7r5 and aortic rings were exposed during 4 days to either non-calcifying medium (Non CM: DMEM-F12 + 0.1% BSA, 1 mM Ca, 1 mM P) or to calcifying medium (CM: DMEM-F12 + 0.1% BSA, 2 mM Ca, 3 mM P) with 0 or 100 μ g/mL of B β Glucans. A7r5 levels of the β -glucan receptor Dectin1 were examined by qPCR and intracellular levels of (1–3) β -D-glucans as indicated in blood chemistries.

Blood chemistries and proteinuria. Human biochemical parameters were measured at the Medicine Laboratory of the Hospital Universitario Central de Asturias using Cobas 8000 (Roche Diagnostics) Module c702, for most parameters, and Module e801 for PTH immunoassay.

For animal studies spectrophotometry and immunoassay (Cobas 8000, Roche Diagnostics) were used to measure serum levels of Ca, P, creatinine and 25-hydroxyvitaminD (25(OH)D). QuantiChrom™ Urea Assay Kit measured Blood Urea Nitrogen (BUN, BioAssay System). ELISA Kits were used to measure rat intact PTH (Immutopics), rat fibroblast growth factor 23 (FGF23; EMD Millipore), rat bone alkaline phosphatase (Biosource), blood and urinary rat TNF α (Abcam plc, Cambridge, UK). Serum human TNF α was measured by bead-based multiplex assay (BiolegendPlex, Biolegend, Germany) analyzed in a FACS Canto II flow cytometer (BD Biosciences) equipped with a FACS Diva 6.5 software, following manufacturers' protocols. Test strips measured urinary protein (SIEMENS MULTISTICK 10SG, Analyticon Biochemistry). The Fungitel™ kit (Associates of Cape Code, Inc) measured serum, plasma and intracellular (1,3) β -D-glucans.

Histological analyses. Rat renal and aortic 5 μ m-paraffin sections were deparaffinized and hydrated. Renal ADAM17 and CD45 (leukocyte infiltration) immunostainings were performed as in⁴⁶ and renal α -klotho with kit-CTS008 (R&D System). For aortic ADAM17 immunofluorescence nuclei were counterstained with Hoechst. For renal ADAM17, sections were counterstained with hematoxylin-eosin. Each slide had its negative control (no primary antibody). Supplementary Table 1 lists primary antibodies and dilutions. Quantifications used ImageJ or Histoscores⁴⁷.

Mitochondrial superoxide production. Circulating human leukocytes and cultured Raw 264.7 cells were washed with PBS, resuspended in 5 μ M superoxide sensitive probe MitoSOX™ (Thermo-Fisher Scientific) in PBS

with either vehicle or the corresponding treatment for 10 min at 37 °C, protected from light and washed twice with PBS. A FACS Canto™II flow cytometer (Becton Dickinson Bioscience) measured superoxide anion levels.

Von kossa staining. Deparaffinized, hydrated rat aorta sections were incubated in 5% silver nitrate before the revealing solution. Slides placed in 2% sodium thiosulfate were counterstained with nuclear fast red.

Total calcium measurements. A 20 mm segment of the abdominal rat aorta proximal to the iliac bifurcation was first grinded in liquid nitrogen and then decalcified with 0.6 N HCl. A7r5 cells, washed thrice with PBS, were decalcified with 0.6 N HCl.

Samples were shaken gently at 4 °C for 24 h. Upon centrifugation, O-cresolphthalein-complexone measured total calcium in supernatants. Pellets were re-suspended in lysis buffer (0.1 N NaOH, 0.1% SDS) for protein extraction and quantification (Lowry method, Bio-Rad).

For rat aortic rings, total calcium was measured as above in cell pellets from RNA extraction (below) and expressed as µg calcium/µg RNA.

Sphingomyelinase2 activity. The Amplex Red Sphingomyelinase assay kit (Molecular Probes, Invitrogen) was used in aorta sections and in A7r5 whole cell extracts.

Quantitative PCR. Total tissue or cell RNA, extracted with TRI reagent (Sigma-Aldrich), was reversed transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative-real time PCR (qPCR) reactions used the Stratagene Mx3005P QPCR System (Agilent Technologies), Fast Start Universal Probe Master (Roche), pre-developed assays (Thermo-Fisher Scientific) and $\Delta\Delta C_t$ quantification⁴⁸.

Statistical analysis. T tests, ANOVA or Kruskal-Wallis with Bonferroni post-hoc test examined statistical differences between groups. Results are expressed as mean \pm SD unless otherwise stated. Statistical analyses used GraphPad Prism, SPSS 17.0 for Windows or R.

Data availability

No datasets were generated or analyzed during the current study.

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Author contributions

M.J.M., C.R., J.B.C.-A. and A.S.D. designed the study; M.V.A., N.C.-L., S.P., A.L.C.-G., P.V., C.U., A.C., C.Q.-C., L.M.-A., J.R.-C. and A.S.D. performed experiments and analyzed results; M.V.A., N.C.-L. made figures; M.V.A., N.C.-L., S.P., C.M.-S., M.J.M., C.R., J.B.C.-A. and A.S.D. drafted and reviewed the manuscript. All authors approved the final version.

Competing interests

The authors declare no competing interests.

Additional information

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